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Correspondence and requests for materials should be addressed to A.K.G. (e-mail: anilg@gg.iitkgp.ernet.in).

Reductive dehalogenation of chlorinated dioxins by an anaerobic bacterium

Michael Bunge*, Lorenz Adrian†, Angelika Kraus‡, Matthias Opel§, Wilhelm G. Lorenz‡, Jan R. Andreesen*, Helmut Görisch† & Ute Lechner*

** Institut für Mikrobiologie and ‡ Institut für Analytik und Umweltchemie, Martin-Luther-Universität Halle-Wittenberg, 06099 Halle, Germany*
† Fachgebiet Technische Biochemie, Institut für Biotechnologie der Technischen Universität Berlin, 13353 Berlin, Germany
§ GfA Gesellschaft für Arbeitsplatz- und Umweltanalytik mbH, 06896 Straach, Germany

Polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDDs and PCDFs) are among the most notorious environmental pollutants. Some congeners, particularly those with lateral chlorine substitutions at positions 2, 3, 7 and 8, are extremely toxic and carcinogenic to humans[1]. One particularly promising mechanism for the detoxification of PCDDs and PCDFs is microbial reductive dechlorination. So far only a limited number of phylogenetically diverse anaerobic bacteria have been found that couple the reductive dehalogenation of chlorinated compounds—the substitution of a chlorine for a hydrogen atom—to energy conservation and growth in a process called dehalorespiration[2]. Microbial dechlorination of PCDDs occurs in sediments and anaerobic mixed cultures from sediments, but the responsible organisms have not yet been identified or isolated. Here we show the presence of a *Dehalococcoides* species in four dioxin-dechlorinating enrichment cultures from a freshwater sediment highly contaminated with PCDDs and PCDFs. We also show that the previously described chlorobenzene-dehalorespiring bacterium *Dehalococcoides* sp. strain CBDB1 (ref. 3) is able to reductively dechlorinate selected dioxin congeners. Reductive dechlorination of 1,2,3,7,8-pentachlorodibenzo-*p*-dioxin (PeCDD) demonstrates that environmentally significant dioxins are attacked by this bacterium.

PCDDs and PCDFs are ubiquitous and recalcitrant environmental pollutants[4,5]. Continuing anthropogenic contamination with PCDDs and PCDFs, formed as unwanted by-products of manufacturing and incineration processes, is of great public concern owing to the compounds' toxicity and tendency to bioaccumulate and biomagnify in wildlife and humans. Natural sources of dioxins include volcanic activities, forest fires, production by biological systems[6,7] and as yet unknown formation processes[8,9]. Because of their high hydrophobicity, dioxins are strongly adsorbed on organic matter and they therefore accumulate in aquatic sediments and soils, where conditions might be anaerobic. The only known biological process leading to a transformation of the highly chlorinated congeners under anaerobic conditions is the microbially mediated reductive dechlorination observed in microcosms or mixed cultures[10–15]. Different sources of PCDDs and PCDFs introduce different complex mixtures of PCDD and PCDF congeners into the environment. The extent to which intrinsic microbes change these source-specific profiles *in situ* is largely unknown, although studies of sediment cores of Lake Ketelmeer, a sedimentation area of the river Rhine in The Netherlands, have shown a change of congener distribution over time[16]. This observation can be taken as an indication that highly chlorinated dioxins are subject to anaerobic dehalogenation processes *in situ*. Our knowledge of the organisms involved in PCDD dechlorination is currently very limited. Until now, no pure culture with the ability to reductively dechlorinate dioxins has been described.

We have previously examined the reductive dehalogenation of selected dioxin congeners by anaerobic mixed cultures[17]. These enrichment cultures were established with various sediment samples from the stream Spittelwasser (Bitterfeld district, Germany), which contains dioxin at concentrations of up to 120,000 pg toxicity equivalents (I-TEQ) per g dry weight. Spiked 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (TeCDD) (50 μM) was converted to a mixture of 1,3- and 2,3-dichlorodibenzo-*p*-dioxin (DiCDD). In previous experiments, the transformation pathways

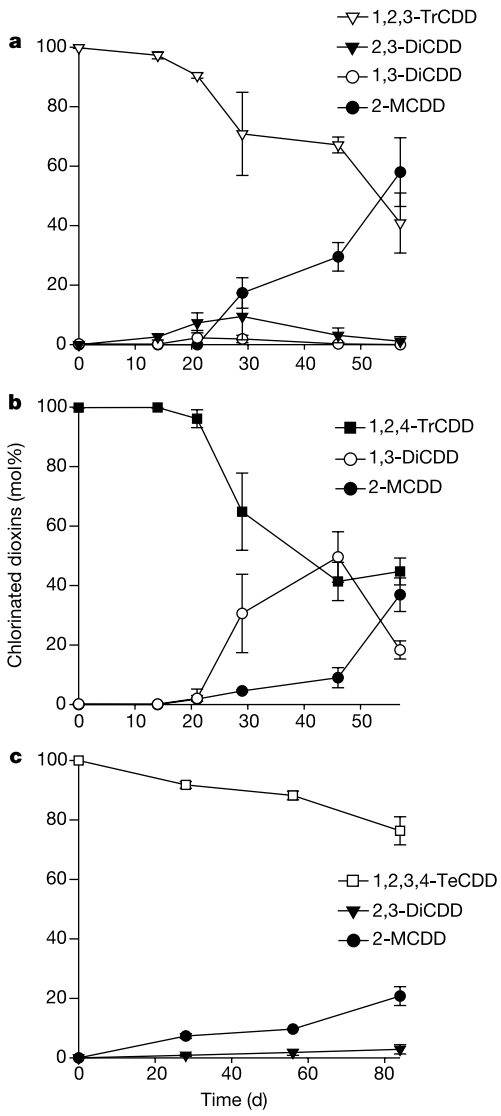

Figure 1 Time course of reductive dechlorination of 25 μM 1,2,3-TrCDD (**a**), 60 μM 1,2,4-TrCDD (**b**) and 46 μM 1,2,3,4-TeCDD (**c**) by *Dehalococcoides* sp. strain CBDB1. Molar distributions of the parent compounds and their dechlorination products are shown. Values are means and s.d. for triplicate samples. No dechlorination products were detected in sterile controls after 75 days (**a**, **b**) and 84 days (**c**).

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were elucidated with subcultures spiked with the possible intermediate trichlorodibenzo-*p*-dioxins (TrCDDs) 1,2,4- and 1,2,3-TrCDD. A comparison of data obtained from the cultures with abiotic treatments (autoclaved and uninoculated controls) clearly demonstrated that the dechlorination was mediated by microorganisms[17].

In this study, the cultures from Spittelwasser sediment were transferred six times (10% v/v each time) into synthetic medium, to obtain sediment-free cultures reproducibly dechlorinating TrCDDs. 1,2,4-TrCDD was dechlorinated to 1,3-DiCDD, and 1,2,3-TrCDD was dechlorinated to 1,3-DiCDD and 2,3-DiCDD as observed in the initial cultures. However, in contrast to the initial cultures, most of the later transfers formed more 2,3- than 1,3-DiCDD from 1,2,3-TrCDD, demonstrating that dechlorination in the peripheral (*peri*)-position was now preferred. In addition, 2-monochlorodibenzo-*p*-dioxin (2-MCDD) was detected as the final dechlorination product of both TrCDDs. Most-probable-number analysis detected only about 10^4 dechlorinating bacteria ml^{-1} in the mixed cultures compared with a total cell number of more than 10^7 cells ml^{-1}. We therefore did not attempt isolation of the dioxin-dechlorinating bacterium by conventional agar-shake dilutions. Instead, a polymerase chain reaction (PCR)-based approach was used to study the presence of several bacteria with known dechlorination potential[2], among them *Dehalococcoides*. This genus comprises two strains with unusual dehalogenation properties: *Dehalococcoides* sp. strain CBDB1 (ref. 3) is the only known bacterium able to dechlorinate chlorinated benzenes, and *D. ethenogenes* strain 195 completely dechlorinates tetrachloroethene to ethene[18]. Using the oligonucleotide primers DET730 and DET1350 targeting the 16S ribosomal DNA (rDNA) of *Dehalococcoides*, PCR products were obtained from the sixth transfers of two 1,2,3- and two 1,2,4-TrCDD-dechlorinating enrichment cultures. Each of the four sequences had a length of about 600 base pairs (bp). They were identical with each other and also with the sequence of *Dehalococcoides* sp. strain CBDB1 (GenBank accession number AF230641), and shared 98.5% identity with the sequence of strain 195 (AF004928.2).

To substantiate an involvement of *Dehalococcoides* in dioxin dechlorination, the capability of strain CBDB1 to transform TrCDDs was studied. Liquid cultures pre-grown anaerobically on trichlorobenzenes were transferred (5% v/v inoculum) into a completely synthetic anaerobic medium[3] containing 5 mM acetate as a carbon source, hydrogen as an electron donor as described[3], and 25–60 μM 1,2,3- or 1,2,4-TrCDD. 1,2,3-TrCDD was dechlorinated to 1,3-DiCDD, 2,3-DiCDD and 2-MCDD, whereas 1,2,4-TrCDD was dechlorinated to 1,3-DiCDD and 2-MCDD (Fig. 1a, b). The time course of the dehalogenation of 1,2,3-TrCDD (Fig. 1a) showed 2,3-DiCDD as the initial and transient dechlorination product first detected after 14 days of incubation. Small amounts of 1,3-DiCDD and 2-MCDD were detectable after 21 days. Whereas concentrations of DiCDD remained at a low level, 2-MCDD concentrations steadily increased with decreasing levels of 1,2,3-TrCDD, leading to the transformation of about 60 mol% TrCDD to 2-MCDD within 57 days. In cultures to which 1,2,4-TrCDD had been added, the extent of reductive dechlorination was still low at day 21 (Fig. 1b). Although the fraction of 1,3-DiCDD increased rapidly thereafter, production of 2-MCDD was low during the first 46 days. At the end of the experiment (57 days), 37 mol% of TrCDD had been converted to 2-MCDD. Neither 1-MCDD nor non-substituted dibenzo-*p*-dioxin was detected throughout the study. Thus, 2-MCDD was the final dechlorination product of both TrCDDs. Autoclaved or uninoculated controls did not show any formation of dechlorinated products.

Three further dioxin congeners available to us were studied for reductive dechlorination by strain CBDB1. 2,3-DiCDD (10 μM) was transformed to 53 mol% 2-MCDD after 28 days of incubation. 1,2,3,4-TeCDD (46 μM) was dechlorinated within 84 days to 3 mol% 2,3-DiCDD and 21 mol% 2-MCDD (Fig. 1c). Traces of

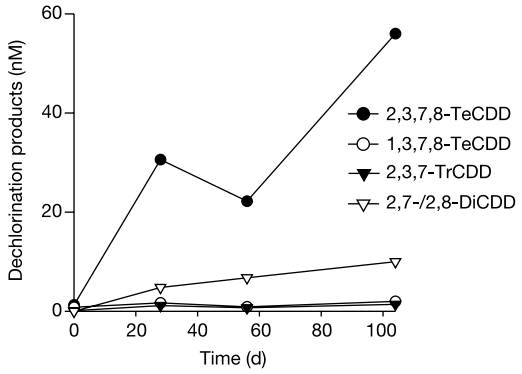

Figure 2 Formation of dechlorination products from 1,2,3,7,8-PeCDD. 1,2,3,7,8-PeCDD was added at a starting concentration of 3 μM. Within 104 days, 2.8 mol% was converted (decrease in PeCDD not shown) to products, which were formed in nanomolar concentrations. Values shown are means for two parallel samples.

1,3-DiCDD were detected only once (after 56 days). The concentrations of 1,2,3- and 1,2,4-TrCDD were below the detection limit throughout the experiment. 1,2,3,7,8-PeCDD (3 μM) was used as a model compound for dioxins chlorinated on both rings and as a representative of the 17 most toxic PCDD and PCDF congeners substituted at positions 2, 3, 7 and 8. The applied concentration (3 μM) was similar to the total PCDD and PCDF concentration (about 6 μM) determined in Spittelwasser sediment[17]. Pure cultures of strain CBDB1 transformed this compound, albeit slowly (2.8 mol% within 104 days), to 2,3,7,8-TeCDD, 2,7-DiCDD or 2,8-DiCDD (see Methods) and small amounts of 1,3,7,8-TeCDD and 2,3,7-TrCDD (Fig. 2). A control with autoclaved inoculum did not show any product formation from PeCDD.

To ensure that the reductive dechlorination of dioxins by strain CBDB1 was independent of the presence of chlorinated benzenes originating from the preculture, the cultures were subcultured sequentially (10% v/v each time) with either 1,2,3-TrCDD or with 1,2,4-TrCDD. The initial inoculum for this experiment originated from a culture spiked with 15 μM 1,2,3-trichlorobenzene and 15 μM 1,2,4-trichlorobenzene as the terminal electron acceptors. The cultures could be successfully transferred into synthetic medium with 1,2,3-TrCDD or 1,2,4-TrCDD at least four times (dilution factor 10,000). The dechlorination pattern remained the same through the four consecutive transfers. Adrian *et al.*[3] previously showed that *Dehalococcoides* sp. strain CBDB1 does not grow in the synthetic medium without an added chlorinated electron acceptor. Because maintenance of the cultures was not dependent on the addition of chlorobenzenes, the data support the hypothesis that PCDD congeners are used as respiratory electron acceptors.

The dioxin dehalogenation reactions observed with strain CBDB1 are summarized in Fig. 3. 1,2,3,4-TeCDD was predominantly transformed by way of 1,2,3-TrCDD, as suggested by the formation of the subsequent intermediate 2,3-DiCDD. This indicates an initial dechlorination at *peri* positions. Dechlorination of spiked 1,2,3-TrCDD by CBDB1 also proceeded preferentially at the *peri* position. However, both DiCDDs are further dechlorinated either at a lateral or peripheral carbon to 2-MCDD as the final dechlorination product. Spiked 1,2,4-TrCDD was strictly *peri*-dechlorinated by the successive removal of chlorines in positions 1 and 4. Such a dechlorination pattern has also been observed in enrichment cultures from sediment layers from the Spittelwasser[17] and the river Saale[14]. The shift from lateral to *peri* dechlorination of 1,2,3-TrCDD observed in the sixth transfers of the mixed cultures from Spittelwasser sediment suggests that *Dehalococcoides* sp. strain CBDB1 represented one subpopulation that was enriched by our culture conditions from a greater diversity of dioxin-dechlorinating bacteria in the sediment community.

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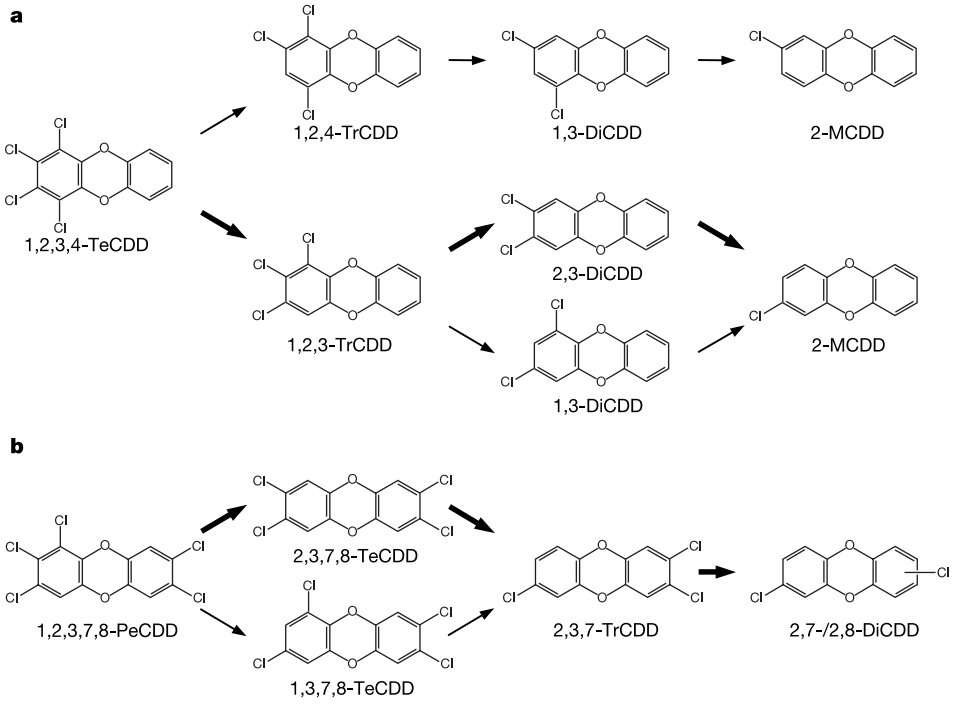

Figure 3 Proposed pathways of reductive dechlorination of spiked 1,2,3,4-TeCDD (**a**) and 1,2,3,7,8-PeCDD (**b**) by a pure culture of *Dehalococcoides* sp. strain CBDB1. The major routes are marked with bold arrows. The results of reductive dechlorination of spiked 1,2,4-TrCDD,1,2,3-TrCDD and 2,3-DiCDD are included in **a**. For visualization purposes, identical structures of 1,3-DiCDD and 2-MCDD are shown inverted.

The removal of *peri*-chlorines from higher chlorinated 2,3,7,8-substituted dioxins involves the risk of forming 2,3,7,8-TeCDD, the most toxic dioxin congener (dioxin activation, upper pathway), as indicated earlier by other authors for sediment-derived mixed cultures[13] and sediment microcosms[15]. However, these same cultures also exhibited dioxin detoxification (lower pathway) by the formation of less harmful TrCDDs and MCDD. This combined *peri*–lateral dechlorination pathway was assigned to non-methanogenic, non-spore-forming bacteria[13].

We used 1,2,3,7,8-PeCDD, the immediate precursor of 2,3,7,8-TeCDD in the upper pathway, to study the dechlorination behaviour of strain CBDB1. It also produced 2,3,7,8-TeCDD but simultaneously the less toxic 1,3,7,8-TeCDD, 2,3,7-TrCDD and 2,7-/2,8-DiCDD. The removal of chlorine atoms from the individual rings of 1,2,3,7,8-PeCDD (as suggested by the products identified) strongly resembles the dechlorination patterns observed with 1,2,3-TrCDD and 2,3-DiCDD. This suggests a transient formation of 2,3,7,8-TeCDD and its further dechlorination through 2,3,7-TrCDD (Fig. 3). With strain CBDB1 it is therefore now possible to analyse and understand the dynamics of microbial transformation of environmentally significant dioxin congeners both in culture and in natural environments. This might help to predict a potential transient hazard involved in the overall detoxification process.

On the basis of 16S rDNA sequence, *Dehalococcoides* sp. strain CBDB1 is affiliated to a major subphylum of the phylum *Chloroflexi* (green non-sulphur bacteria). The tetrachloroethene-dehalorespiring bacterium *Dehalococcoides ethenogenes* strain 195 and *Dehalococcoides* sp. strain CBDB1 are so far the only isolated and cultivated representatives of the '*Dehalococcoides*' subphylum[3,18]. For both strains, growth on fermentable substrates or the use of non-halogenated electron acceptors was not observed. Available 16S rDNA sequence information indicates the frequent presence of *Dehalococcoides*-related bacteria in mixed cultures that dehalogenate a variety of chlorinated substances[19–22]. Additionally, two bacteria with distant relatedness to *Dehalococcoides* were described recently as being responsible for the dechlorination of polychlorinated biphenyls[23,24]. The high genetic potential of this genus for dechlorination reactions is evident from the genome sequence of *Dehalococcoides ethenogenes* (see the TIGR website at http://www.tigr.org/tdb/mdb/mdbinprogress.html). At least 15 reductive dehalogenase-homologue genes were detected in the genome, suggesting that different enzymes are involved in the reductive dehalogenation of different substrates. *Dehalococcoides* is thought to be well adapted for anaerobic reductive dehalogenation, which might be an ancient process for the turnover of naturally produced organohalogens. Owing to their dehalogenation potential, indigenous and introduced organisms of the *Dehalococcoides* cluster are an important addition to the arsenal of organochlorine-transforming microorganisms[2] that are potentially applicable to the bioremediation of contaminated sites containing anthropogenic PCDD. □

Methods

Analytical techniques

MCDD, DiCDD and non-chlorinated dibenzo-*p*-dioxin were analysed from the headspace of the cultures by solid-phase microextraction (SPME, 100 μm polydimethylsiloxane coated fibres; Supelco). The conventional method, including freeze-drying and concentration by evaporation[14], yielded from the same samples only up to 21% of the 2-MCDD concentration compared with SPME, whereas the DiCDDs were found at similar concentrations. Samples were preconditioned for 2 h at 54 °C; fibres were equilibrated for 35 min at 54 °C and desorbed for 195 s (injection port: 260 °C) followed by splitless injection (0.7 min). The Shimadzu GC14 A/FID gas chromatograph was equipped with a DB-608 capillary column (30 m × 0.331 mm internal diameter, 0.5 μm film thickness). A six-level external calibration curve from the headspace over minimal medium (amended with the respective dioxin concentrations ranging from 0.78 to 25 μM) was generated. On the basis of the similar molar response within a given homologue group[25], dichlorinated dioxins were quantified with 2,7-DiCDD as a calibration standard. After SPME analysis, the liquid cultures were extracted and analysed for the respective tetrachlorinated and trichlorinated congeners as described[14]. Identification of monochlorodioxins and dichlorodioxins was confirmed on a gas chromatograph with mass-selective detector (GC/MSD; HP6890/HP5973) from mass spectra and the relative retention of authentic standards[14].

For every data point, two or three individual 3-ml cultures were harvested. Heterogeneities of absolute dioxin concentrations, which might have been due to slight differences in starting concentrations, recovery efficiencies and sorption onto differing cell numbers, were normalized by the expression of each congener as mol% of the sum of all congeners detected. 1,2,3,7,8-PeCDD and its dechlorination products were analysed on a

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GC/MSD after extraction of the 3-ml cultures with toluene, gentle concentration and separation on a SP-2331 column (60 m × 0.25 mm internal diameter, 0.2 μm film thickness) in accordance with standard conditions for dioxin analyses[26]. The relative retention times and response factors were determined by analysing calibration mixtures containing five native congeners (1,2,3,7,8-PeCDD, 2,3,7,8-TeCDD, 1,2,4-TrCDD, 2,7-DiCDD and 1-MCDD) and five $^{13}C_{12}$-labelled internal standards (1,2,3,7,8-PeCDD, 2,3,7,8-TeCDD, 1,3,6,8-TeCDD, 2,3,7-TrCDD and 2,8-DiCDF). Before extraction, the five $^{13}C_{12}$-labelled congeners were added and the recovery efficiency compared with that of $^{13}C_6$-1,2,3,4-TeCDD was determined. The recovery efficiency ranged between 75% and 100%. 2,3-DiCDD and 2,7-DiCDD could be identified by relative retention compared with $^{13}C_{12}$-2,3,7-TrCDD, but 2,7-DiCDD and 2,8-DiCDD were not expected to be separated under these conditions.

Cultivation

Chlorinated dibenzo-*p*-dioxins (amchro, Hattersheim, Germany) were dissolved in acetone and added to each cultivation tube; the solvent was evaporated by using 20% CO_2/80% N_2. The synthetic culture medium[3] was prepared with strict anaerobic techniques. *Dehalococcoides* sp. strain CBDB1 was cultivated under anaerobic conditions with a gas phase of 20% CO_2/80% N_2 in several parallel cultures in 15-ml Hungate tubes (Bellco Glass, Inc., Vineland, New Jersey, USA) containing 3 ml of culture volume sealed with thick butyl rubber stoppers (Ochs Glasgerätebau, Bovenden, Germany), which were best suited to the maintenance of highly reduced conditions over long cultivation times, essential for the growth of strain CBDB1. For some experiments Teflon disks were placed below the septa to reduce potential sorption on the stoppers. Recovery of total dioxins decreased over time. After 4 weeks of incubation, recovery ranged between 54% and 92% without Teflon coats, and between 38% and 76% with the coats. The cultures were supplied with 5 mM acetate and hydrogen (2.5 ml injected into the headspace of the Hungate tubes, corresponding to an approximate dissolved hydrogen concentration of 0.1 mM). The consecutive transfers into fresh medium with acetate, hydrogen and TrCDDs were performed every 24 days. The cultures were incubated at 30 °C in the dark and agitated at 115 r.p.m. A comparison with non-agitated cultures demonstrated that agitation promoted dechlorination. For example, after 56 days, 60 mol% of the 1,2,3-TrCDD was converted in an agitated culture and only 40 mol% in a static culture. Controls were established with autoclaved inocula, and without inoculum.

PCR detection and sequence analysis

Community DNA, extracted from dioxin-dechlorinating enrichment cultures with the use of standard methods, was used as a template for almost complete 16S rDNA amplification with the universal primer pair fD1 and rP2. PCR amplification and DNA sequencing methods have been described in detail previously[27]. Amplified products from the initial PCR were then used as templates in the second amplification with *Dehalococcoides*-targeted primers DET730 (5′-GCGGTTTTCTAGGTTGTC-3′) and DET1350 (5′-CACCTTGCTGATATGCGG-3′), which were specifically designed with ARB software[28]. Obtained amplicons were sequenced entirely in both directions and analysed as described[27].

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Correspondence and requests for materials should be addressed to M.B. (e-mail: m.bunge@mikrobiologie.uni-halle.de).

Directional postcopulatory sexual selection revealed by artificial insemination

Jonathan P. Evans, Lorenzo Zane, Samuela Francescato & Andrea Pilastro

Department of Biology, University of Padova, via U. Bassi 58/B, Padova, 1-35131, Italy

Postcopulatory sexual selection comprises both sperm competition, where the sperm from different males compete for fertilization[1], and cryptic female choice, where females bias sperm use in favour of particular males[2]. Despite intense current interest in both processes as potential agents of directional sexual selection[3], few studies have attributed the success of attractive males to events that occur exclusively after insemination. This is because the interactions between pre- and post-insemination episodes of sexual selection can be important sources of variation in paternity[4]. The use of artificial insemination overcomes this difficulty because it controls for variation in male fertilization success attributable to the female's perception of male quality, as well as effects due to mating order and the relative contribution of sperm from competing males[5]. Here, we adopt this technique and show that in guppies, when equal numbers of sperm from two males compete for fertilization, relatively colourful individuals

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